Nitric Oxide Modulates Synaptic Vesicle Docking/Fusion Reactions

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Summary

Nitric oxide (NO) stimulates calcium-independent neurotransmitter release from synaptosomes. NO-stimulated release was found to be inhibited by Botulinum neurotoxins that inactivate the core complex of synaptic proteins involved in the docking and fusion of synaptic vesicles. In experiments using recombinant proteins, NO donors increased formation of the VAMP/SNAP-25/syntaxin 1a core complex and inhibited the binding of n-sec1 to syntaxin 1a. The combined effects of these activities is predicted to promote vesicle docking/fusion. The sulfhydryl reagent NEM inhibited the binding of n-sec1 to syntaxin 1a, while β-ME could reverse the NO-enhanced association of VAMP/SNAP-25/syntaxin 1a. These data suggest that post-translational modification of sulfhydryl groups by a nitrogen monoxide (likely to be NO^+) alters the synaptic protein interactions that regulate neurotransmitter release and synaptic plasticity.

Introduction

Synaptic transmission by the regulated exocytotic release of neurotransmitters is fundamental to signaling in the central nervous system. Studies of synaptic proteins have led to a pathway of protein-protein interactions proposed to correspond to sequential aspects of docking, activation, and fusion of synaptic vesicles with target membranes (Söllner et al., 1993a, 1993b; Pevsner et al., 1994a, 1994b). One working hypothesis proposes that two proteins on the synaptic vesicle, VAMP (also known as synaptobrevin) and synaptotagmin (v-SNAREs), interact with two molecules on the plasma membrane, SNAP-25 and syntaxin (t-SNAREs), to form a 7S complex. Since the heterotrimer complex of VAMP, syntaxin, and SNAP-25 is very stable, even in the presence of SDS, it is known as the core complex. Syntaxin is proposed to be associated with a soluble factor, n-sec1, prior to and perhaps during formation of the 7S complex. Additional complexes are generated as soluble proteins (NSF and α-SNAP) add to the 7S complex with a corresponding loss of synaptotagmin. While the final steps that lead directly to membrane fusion are less well-defined, a rearrangement of the core complex proteins is likely to be important.

In previous work, we reported the stimulation of synaptic vesicle exocytosis by the membrane-permeant messenger, NO (Meffert et al., 1994). NO is synthesized in cells of both the central and peripheral nervous systems and has been implicated in several forms of synaptic plasticity. In contrast to the reversible receptor binding of most neurotransmitters, NO has been demonstrated to form covalent linkages and redox interactions with intracellular proteins (Stamler, 1994). Our prior work demonstrated that NO did not require an elevation of cytosolic calcium for the stimulation of secretion. The ability of NO to stimulate vesicle release without the aid of calcium suggested that it could be acting downstream or parallel to the proteins that confer calcium-dependence to neurotransmitter release. Alternatively, one could hypothesize that NO might obviate the need for synaptic proteins altogether, perhaps using a mechanism such as an alteration of membrane fluidity to achieve vesicle fusion. To differentiate between these hypotheses, we utilized botulinum neurotoxins that specifically cleave essential protein components of the neuroexocytotic apparatus (Schiavo et al., 1992; Montecucco and Schiavo, 1993). Using these toxins, we found that the synaptic proteins VAMP, syntaxin, and SNAP-25 are neccesary for NO-stimulated secretion. Thus, while apparently circumventing a calcium-dependent step, NO nonetheless requires the conventional protein exocytotic machinery.

Understanding the molecular mechanisms that mediate synaptic vesicle docking and fusion is especially interesting because the modulation of these processes, and consequently of neurotransmitter release, is expected to contribute to some forms of synaptic plasticity. Even changes in the quantities of various vesicle proteins have been shown to alter presynaptic activity (Hunt et al., 1994; Broadie et al., 1995; DeBello et al., 1995). Therefore, it might be expected that a biochemical modification affecting the affinity of synaptic proteins for each other or their availability could have similar implications for neurotransmitter release. To investigate whether the ability of NO to stimulate synaptic vesicle exocytosis might be due to direct modification of synaptic proteins, we studied the effects of NO on the binding interactions of the purified recombinant synaptic proteins: n-sec1, syntaxin 1a, VAMP 2, and SNAP-25. We now report that a biochemical modification produced by NO, most likely to be S-nitrosylation, alters the affinities with which these proteins associate to form vesicle complexes.

Results

Effects of BoNTs on NO-Stimulated Vesicle Release

To test whether NO-stimulated neurotransmitter release required intact synaptic transmission protein machinery, we used BoNTs to selectively cleave proteins that have previously been demonstrated to be essential for calcium-dependent neuroexocytosis (McMahon et al., 1992; Blasi et al., 1993a, 1993b). Stimulation of exocytosis by KCl or NO can be monitored by measuring uptake of the fluorescent dye FM1-43 to synaptic vesicles or loss of the fluorescence from vesicles preloaded

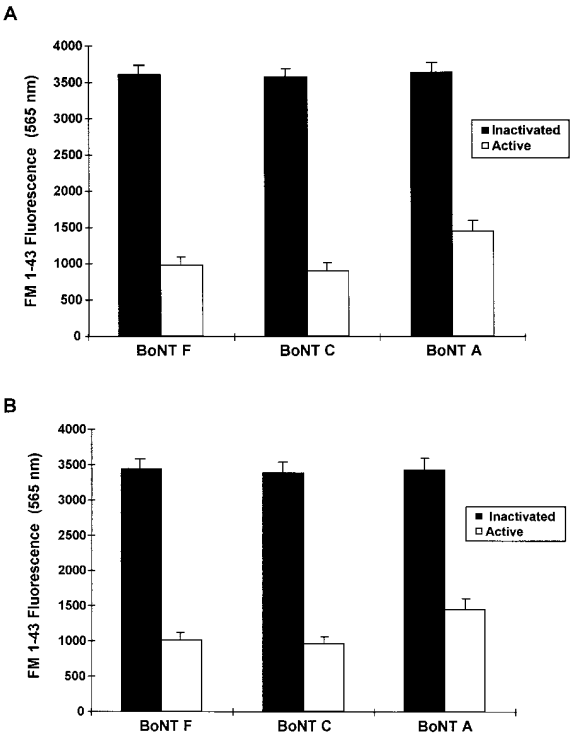

Figure 1. BoNTs Inhibit NO-Stimulated FM1-43 Uptake

Uptake of FM1-43 fluorescence was measured after incubation with heat-inactivated (closed) or active (open) BoNT F, BoNT C, or BoNT A. Fluorescence uptake was determined from starting values of the raw data traces after subtracting a background of slowly declining fluorescence (see Experimental Procedures). In (A), loading of FM1-43 following toxin incubation was stimulated by depolarization with 40 mM KCl and in (B) by addition of a NO donor, 500 μM SNP. Each bar shown was obtained by averaging the starting fluorescence values from three experimental records; all error bars represent one SEM. Following loading, decreases in FM1-43 fluorescence (release of neurotransmitter) could be stimulated by either NO or KCl in samples treated with inactivated BoNT (data not shown). Small decreases in fluorescence could also be stimulated from samples incubated with active BoNT, indicating that some of the residual fluorescence loading in samples treated with active toxin is due to incomplete inhibition of vesicle cycling.

with dye. BoNTs require a long incubation (90 min at 37°C) with synaptosomes, to be bound, internalized, and for the enzymatically active light chain to gain access to the cytosol (McMahon et al., 1992; Blasi et al., 1993a; Yamasaki et al., 1994). FM1-43 preloaded into synaptosomes would surely be slowly released during an incubation of this time and temperature. Therefore, we utilized the ability of NO or depolarization with potassium chloride (KCl) to stimulate FM1-43 loading in toxin-treated synaptosomes, as a measure of their ability to induce vesicle exocytosis. This approach is based on the fact that loading of releasable FM1-43 fluorescence requires a cycle of vesicle exocytosis (Betz et al., 1992; Ryan et al., 1993; Meffert et al., 1994).

BoNT F (open bars), which cleaves VAMP, inhibited equally the ability of either KCl (Figure 1A) or NO (Figure 1B) to stimulate vesicle exocytosis, as measured by loading of FM1-43 fluorescence. Heat-inactivated BoNT F (closed bars) did not inhibit NO or KCl-stimulated loading, and was not significantly different than incubation without toxin (data not shown). Similar results were found using BoNT C that cleaves syntaxin, or BoNT A that cleaves SNAP-25 (Figure 1). In agreement with previously published data on release from synaptosomes, BoNT C and BoNT F were both more effective at inhibiting vesicle exocytosis than was BoNT A (McMahon et al., 1992; Yamasaki et al., 1994). Other NO donors, including S-nitrosocysteine (S-NC) and acidified sodium nitrite (SN), produced similar effects (data not shown). To allow direct comparison of the effects of different BoNTs, only the data with a single donor (SNP) is shown.

NO Potentiates SNAP-25 and VAMP2 Binding to Syntaxin 1a

We investigated the effect of NO on the ability of recombinant VAMP2, SNAP-25, and syntaxin 1a to form the core complex known to be critical for neurotransmitter release (Hayashi et al., 1994; Pevsner et al., 1994b). A fusion protein consisting of the cytoplasmic domain of syntaxin 1a coupled to glutathione-S-transferase (GST) was attached to glutathione agarose beads and used in an in vitro-binding assay. We then measured the binding of increasing concentrations of VAMP2 to syntaxin 1a/GST beads in the presence of a constant amount of soluble SNAP-25 (1 μM) (Figure 2). In the absence of NO (Figure 2A) half-maximal saturation of VAMP binding to SNAP-25/syntaxin 1a occurred at ~1.5 μM. Treatment of the proteins with of the NO donor S-NC (500 μM) lowered the EC_{50} of VAMP binding to SNAP-25/syntaxin to ~0.9 μM (Figure 2B); 1 mM S-NC lowered the EC_{50} further, to ~0.5 μM (Figure 2C). Other NO donors, including SNP, acidified sodium nitrite, S-nitrosoglutathione (S-NG), and a saturated solution of NO gas, had similar effects. The amount of VAMP bound to SNAP-25/syntaxin at saturating VAMP concentrations (5 μM) was not changed by NO. Band intensity measured as phosphoimage pixel values for 5 μM VAMP binding ± 1 mM S-NC were not significantly different ($n = 3$; $p = 0.104$, using a two-tailed t-test).

NO donors had no effect on the pairwise binding of SNAP-25 to syntaxin 1a/GST, without the presence of VAMP (data not shown). NO also did not appear to potentiate VAMP binding to syntaxin 1a/GST without the presence of SNAP-25, although this association has a low affinity (Calakos et al., 1994; Pevsner et al., 1994b). It may be that NO is only capable of affecting binding in the conformation of this heterotrimer.

NO Inhibits n-sec1 Binding to Syntaxin 1a

n-sec1 is involved in regulating the interactions of proteins in the core complex. The effect of NO on the association of n-sec1 with syntaxin 1a was examined using a variety of NO donors (as above). The binding of increasing concentrations of recombinant n-sec1 to syntaxin 1a/GST beads was measured in the presence or absence of NO (Figure 3). In the absence of NO, the binding of n-sec1 to syntaxin 1a/GST beads was saturable with an EC_{50} of ~40 nM. Treatment with 500 μM SNP raised the EC_{50} to ~100 nM (Figure 3B); 1 mM SNP raised the EC_{50} further for n-sec1 binding to syntaxin 1a to ~200

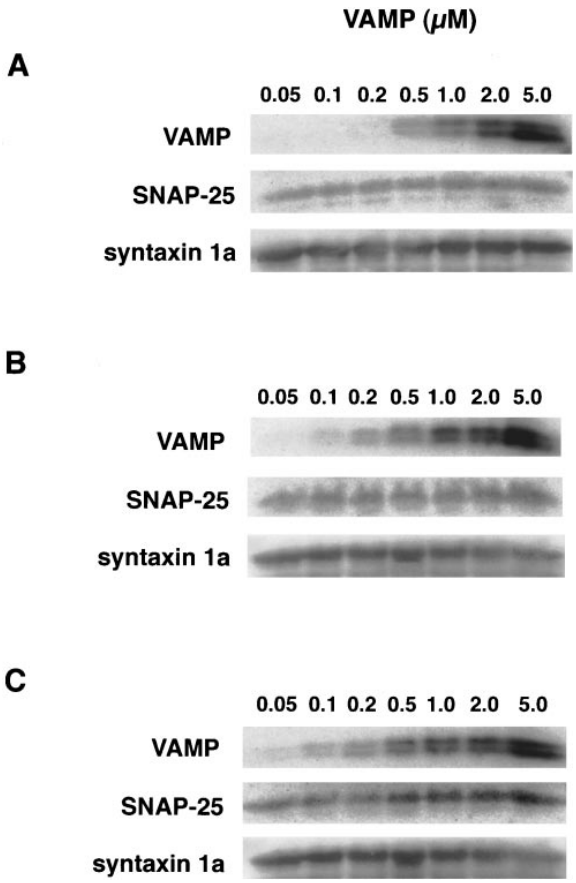

Figure 2. NO Increases Formation of the VAMP 2/SNAP-25/Syntaxin 1a Heterotrimer

Soluble GST-fusion proteins were incubated with syntaxin 1a/GST immobilized on glutathione beads, and bound proteins were detected by Western blotting with ^{125}I-labeled secondary antibody. Syntaxin 1a/GST beads (0.4 μM) were incubated with SNAP-25 (1 μM) and increasing concentrations of VAMP2.

(A) Binding of VAMP2 to SNAP-25/syntaxin 1a in the absence of NO.

(B) Binding of VAMP2 to SNAP-25/syntaxin 1a in the presence of 500 μM SNP.

(C) Binding of VAMP2 to SNAP-25/syntaxin 1a in the presence of 1 mM SNP.

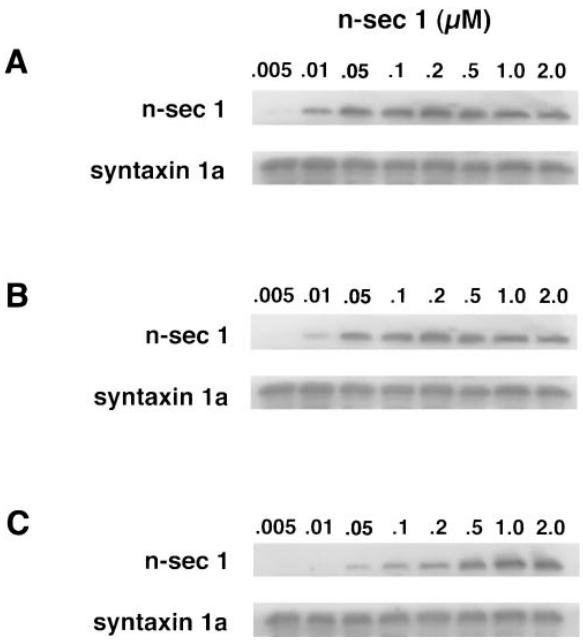

Figure 3. NO Inhibits Binding of n-sec1 to Syntaxin 1a

Increasing concentrations of soluble recombinant n-sec1 were incubated with syntaxin 1a/GST beads (0.3 μM), bound n-sec1 was detected by Western blotting with ^{125}I-labeled secondary antibody.

(A) Binding of n-sec1 to syntaxin 1a in the absence of NO.

(B) Binding of n-sec1 to syntaxin 1a in the presence of 500 μM S-NC.

(C) Binding of n-sec1 to syntaxin 1a in the presence of 1 mM S-NC.

nM (Figure 3C). As with its effect on the VAMP/SNAP-25/syntaxin 1a association, NO did not appear to alter the saturating concentration of n-sec1 bound by syntaxin 1a. Pixel values for 2.0 μM n-sec1 binding ± 1 mM SNP were not significantly different ($n = 4$; $p = 0.147$).

Modification of Sulfhydryl Groups

Previous work using [^{14}C]iodoacetamide to label-free sulfhydryl residues has demonstrated that NO is capable of S-nitrosylating neuronal proteins, including SNAP-25, in intact synaptosomes and brain slices (Hess et al., 1994). We verified this under our conditions with purified synaptic proteins using dithionitrobenzoic acid (DTNB) to monitor free sulfhydryls (Ellman, 1959) before and after exposure to NO gas. SNAP-25, syntaxin 1a, and n-sec1, as well as several other vesicle proteins, all demonstrated a decreased number of free sulfhydryl groups following treatment with NO, indicating that they had undergone S-nitrosylation (data not shown). This S-nitrosylation lasts several hours under the assay conditions of purified proteins kept on ice. However, these conditions bear little similarity to an in-vivo environment where the half-life of an S-nitroso bond would be much less predictable.

Our in vitro-binding assays consisted of purified proteins. Thus, we reasoned that NO must be directly affecting the synaptic proteins in these assays, since no other intermediates (e.g., kinases, phosphatases, etc.) are present. To investigate the hypothesis that NO might be modulating the protein-protein interactions via S-nitrosylation, we examined the effects of several known sulfhydryl modifiying agents on these same interactions. N-ethylmaleimide (NEM) is a sulfhydryl-alkylating agent that binds specifically and permanently to free sulfhydryl groups and can thereby either mimic or occlude (by prior treatment) the effects of NO on protein sulfhydryl groups (Dimmeler and Brune, 1993; Kruszyna et al., 1993; Bolotina et al., 1994). NEM is a much more bulky group than NO, and its potential to mimic versus block the actions of NO is believed to be dependent on the conformations of the particular proteins involved. The effect of NEM (3 mM) on the association of n-sec1 with syntaxin 1a/GST beads is shown in Figure 4. Our experiments revealed that NEM, like NO, is capable of inhibiting the interaction of n-sec1 with syntaxin 1a. The presence of NEM shifted the EC_{50} from ~40 nM to ~300 nM, similar to the effect of NO donors.

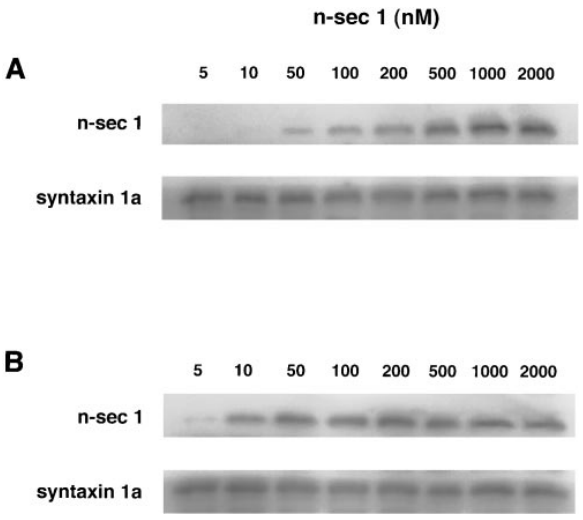

Figure 4. Sulfhydryl Alkylation by NEM Inhibits Binding of n-sec1 to Syntaxin 1a

Increasing concentrations of soluble recombinant n-sec1 were incubated with syntaxin 1a/GST beads (0.3 μM) in the presence (A) and absence (B) of 3 mM NEM. Bound n-sec1 was detected by Western blotting with ^{125}I-labeled secondary antibody.

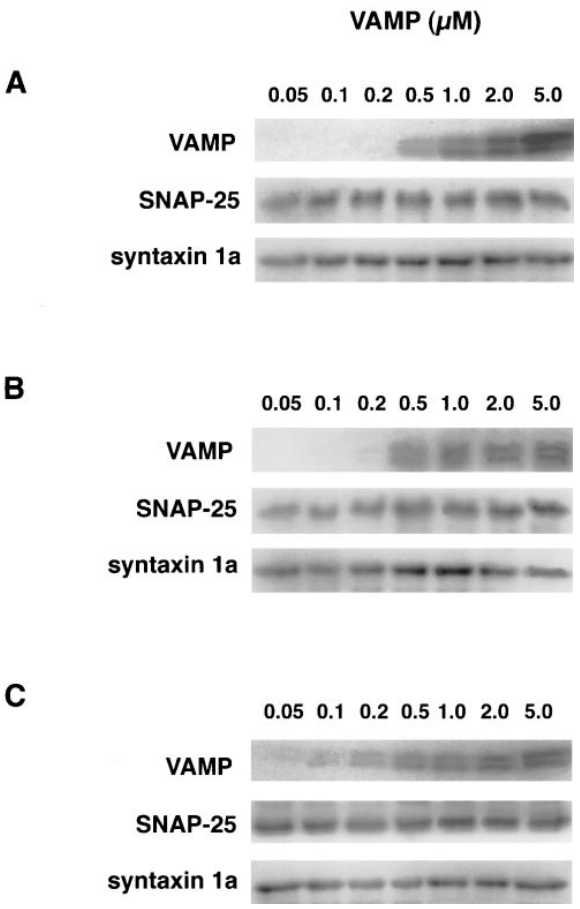

Figure 5. β-ME Reverses the Effects of NO on VAMP/SNAP-25/Syntaxin 1a Complex

Soluble SNAP-25 (1 μM) and increasing concentrations of VAMP2 were incubated with syntaxin 1a/GST beads (0.4 μM), and bound proteins were detected by Western blotting with ^{125}I-labeled secondary antibody.

(A) Binding of VAMP2 to SNAP-25/syntaxin 1a in the absence of NO or β-ME.

(B) Binding of VAMP2 to SNAP-25/syntaxin 1a after exposure to 1 mM S-NC followed by treatment with β-ME (500 μM).

(C) Binding of VAMP2 to SNAP-25/syntaxin 1a after exposure to 1 mM S-NC.

The effect of NEM was specific to the interaction between n-sec1 and syntaxin 1a. Treatment with NEM during the binding incubation was found to have no effect on the association of VAMP2, SNAP-25, and syntaxin 1a/GST. When proteins were treated with NEM prior to a binding incubation in the presence of NO, NEM did appear able to inhibit the effects of NO on heterotrimer association. However, these results are somewhat difficult to interpret, since NEM could simply be blocking the effects of NO by sterric hindrance or a conformational change. Accordingly, we carried out experiments using another sulfhydryl reagent, β-Mercaptoethanol (β-ME), that is capable of displacing NO from sulfhydryl groups (Caselli et al., 1994), and therefore might be able to reverse the effect of NO on the VAMP/SNAP-25/syntaxin 1a association if this effect involved S-nitrosylation. β-ME partially reversed the potentiation of the VAMP/SNAP-25/syntaxin 1a association by 1 mM NO, although the binding was not completely reversed to control (without NO) levels (Figure 5). Samples treated with only NO (Figure 5C) had an EC_{50} for VAMP binding to SNAP-25/syntaxin 1a of ~0.5 μM, samples exposed to NO followed by β-ME treatment had an of EC_{50} ~1.0 μM (Figure 5B), untreated samples had an EC_{50} of ~1.5 μM (Figure 5A).

Native SDS-Resistant Complex Formation Is Enhanced by NO

A stable high molecular weight SDS-resistant complex containing SNAP-25, VAMP, and syntaxin can be detected in rat brain. This complex can be dissociated by boiling and has an M_r ~80 kDa (Hayashi et al., 1994). Synaptic proteins in mammalian neurons may be subject to modifications and associations that are not present in bacterially expressed fusion proteins; therefore, we were interested to examine the effect of NO on the formation of this native complex. Rat hippocampal synaptosomes or diced hippocampi were prepared in a saline solution containing 1 mM EGTA, exposed briefly to NO, and then homogenized in low SDS sample buffer (see Experimental Procedures). Figure 6 shows the complex in diced hippocampi after SDS–PAGE analysis and immunoblotting for VAMP. Treatment with NO donors (S-NC, Figure 6) resulted in a small but significant increase in the amount of SDS-resistant complex detected by quantitative phosphorimaging ($n = 4$; $p = 0.038$, using a two-tailed t-test). Boiling of samples resulted in dissociation of the complex both with and without NO treatment. Similar results were obtained with hippocampal synaptosomes, except that less total protein was required to detect similar amounts of SDS-resistant complex. NO also enhanced complex formation when detected by immunoblotting for syntaxin (data not shown).

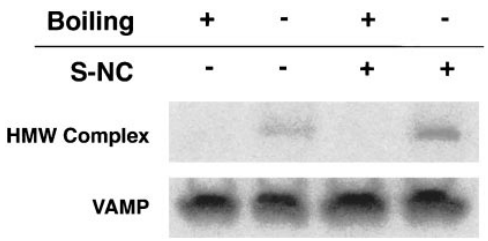

Figure 6. NO Increases Formation of Native SDS-Resistant Complex in Hippocampal Homogenates

Hippocampi were diced in BSS plus 1 mM EGTA ± S-NC (as indicated in figure) and homogenized in sample buffer (0.5 mg/ml). Samples were either boiled (+) or incubated at 37°C (−) prior to analysis on SDS gels. The high molecular weight (HMW) complex (~80 kDa) or SDS-resistant complex is only disassociated after boiling. Immunoblots were probed with antibodies to VAMP. All lanes are from one gel.

Discussion

Understanding the mechanism(s) responsible for the regulation of neurotransmitter release remains one of the fundamental gaps in our knowledge of synaptic transmission. As the protein complexes responsible for vesicle docking and fusion have become more fully elucidated, experiments have been performed to examine posttranslational modification of these proteins as possible targets for the modulation of the number and release probability of synaptic vesicles. Previous work has shown that several proteins in the docking/fusion complexes can be modified by phosphorylation (Bennett et al., 1993; Davletov et al., 1993; Popoli, 1993; Rubenstein et al., 1993; Fykse et al., 1995). However, phosphorylation has not yet been shown to alter the binding interactions of proteins in the docking/fusion complexes. Thus, the ability of posttranslational modification by NO to affect protein associations in the docking/fusion complex represents a novel potential mechanism for the regulation of neurotransmitter release and synaptic plasticity.

We chose to examine the effect of NO on the association of the SNARE proteins because they are hypothesized to form the core complex of synaptic vesicle docking and fusion. Recent evidence suggests that components of the core complex (VAMP and syntaxin) function not only in docking but also in downstream vesicle fusion events. Syntaxin, in particular, has been proposed to be required for the actual fusion event between vesicle and plasma membrane; both spontaneous and induced vesicle fusion fail to occur in the absence of syntaxin (Hunt et al., 1994; Broadie et al., 1995). Potentiation of the VAMP/SNAP-25/syntaxin heterotrimer by NO (Figure 2) might therefore be expected to have consequences not only for docking but also for membrane fusion. We hypothesize that potentiation of this complex could in part acount for the ability of NO to stimulate vesicle fusion. A role for n-sec1 as a modulator of synaptic vesicle docking has been suggested by its ability to inhibit binding of SNAP-25 and VAMP to syntaxin 1a in vitro (Pevsner et al., 1994b). Our finding that NO inhibits the association of n-sec1 with syntaxin (Figure 3) might enhance formation of the heterotrimer in vivo further, since the binding and release of syntaxin by n-sec1 may be a neccesary step on the path to heterotrimer formation (Schulze et al., 1994; Pevsner et al., 1994b). Thus, while NO produced changes in protein-protein interactions that are opposite in nature, the combination of the effects of NO might be predicted to advance synaptic protein interactions leading to vesicle fusion. NO may also have effects on additional synaptic proteins that we have not yet investigated, and it may be the combined effects of all these interactions that allow NO to stimulate neurotransmitter release without requiring an elevation in intracellular calcium.

The inhibition of the n-sec1/syntaxin 1a complex and potentiation of the VAMP/SNAP-25/syntaxin 1a complex must be due to direct effects of NO-related activity on these proteins, since the experiments were carried out with purified proteins in a cell-free system. Transition metals, such as the centers of iron sulfur clusters and heme proteins, are well-known sites for redox and coordinative interactions of NO. Covalent modification by NO may occur at a variety of nucleophilic centers, including tyrosine residues, deoxyribonucleic acids, and thiol groups (Lei et al., 1992; Nguyen et al., 1992; van der Vliet et al., 1995). The greater prevalence and reactivity of thiols results in a propensity for S-nitrosothiol formation over adducts with other biological nucleophiles (Stamler et al., 1992); S-nitrosylation has been shown to be important in a wide variety of experimental systems, including the regulation of smooth muscle relaxation by activation of calcium-dependent potassium channels, the inhibition of adenylyl cyclase, and the inactivation of protein kinase C (Gopalakrishna et al., 1993; Bolotina et al., 1994; Duhe et al., 1994). The inhibition of the n-sec1/syntaxin 1a interaction is consistent with the propensity for S-nitrosylation to inhibit protein action (McDonald and Murad, 1996). Our experiments with the sulfhydryl alkylating and reducing agents, NEM and β-ME, indicate that the binding affinities in both n-sec1/syntaxin 1a and VAMP/SNAP-25/syntaxin 1a complexes can be altered by the modification of thiol residues (Figures 4 and 5). This suggests that S-nitrosothiol formation could account for the effects of NO on these complexes. Which thiol residues are critical for altering binding affinities and which residues can be modified when the proteins are associated in complexes remains unclear. Numerous cysteines are present in putative NO targets; n-sec1 has seven cysteines (Pevsner et al., 1994a) and SNAP-25 has four cysteines (Oyler et al., 1989), for example. The identity and function of additional proteins and lipids present in synaptosomes and not in the reconstituted complex needs to be defined before the relative contributions of each protein to the effect of NO can be rigorously determined.

The reaction of the nitrogen monoxide-free radical itself with thiol groups may be relatively unfavorable under physiological conditions. Instead, alternative redox states of nitrogen monoxide, such as NO^+, may be responsible for the direct reactions with thiol groups (Pryor et al., 1982; Lipton et al., 1993; McDonald and Murad, 1996). A necessity for NO^+ (or some other intermediate nitrosating agent) could acount for the higher concentrations of nitric oxide donors required in our experiments. Whatever the mechanism for formation,

once formed, thionitrites lack the high reactivity of the NO-free radical and tend to be more stable (Stamler et al., 1992). How long an individual S-nitrosyl modification might be expected to last depends on both the redox environment and on the nature of the thiol group (RS) itself, (e.g., the electron-withdrawing nature of the R). The S-nitrosylation of critical thiol residues could alter intramolecular hydrogen bonding or electrostatic interactions to achieve structural changes making the association of n-sec1/syntaxin 1a less favorable and enhancing formation of the VAMP/SNAP-25/syntaxin 1a complex. S-nitrosylated thiol residues also have a higher potential for reacting with neighboring thiol groups (with NO as a leaving group) to form disulfide bridges (Arnelle and Stamler, 1995). From our data we are unable to determine whether S-nitrosylation or the formation of disulfide bridges or both are responsible for the alterations in synaptic protein-binding affinities incurred by NO.

Thiol groups are known to be targets for post-translational protein modification in mammalian cells (e.g., palmitylation, myristoylation, ADP-ribosylation). Our finding that NO induced a statistically significant increase in the amount of SDS-resistant complex in diced hippocampi (Figure 6) or synaptosomes suggests that NO may affect synaptic proteins in mammalian cells in the same manner as bacterially expressed fusion proteins. It seems likely that even small changes in the formation of this core complex could have important consequences for vesicle docking and fusion. In addition, the amount of VAMP, SNAP-25, and syntaxin associated in the SDS-resistant complex appears to be small when compared to the total pool of these proteins, as expected if only a small percent of vesicles are docked. The density of the VAMP bands in Figure 6, for example, are more than twenty times the density of the SDS-resistant bands probed with the same anti-VAMP antibody (all bands are from the same gel). This means that we are not looking at all of the VAMP, syntaxin, and SNAP-25 that might be affected by NO, but only the small subset that happens to be captured in the SDS-resistant complex at any given time. Nonetheless, these results indicate that NO continues to have a potentiating effect on the formation of the core docking/fusion complex in the more intricate in vivo environment where protein modifications, additional binding proteins, lipids, and reducing agents are certainly present.

Many components of the synaptic vesicle docking and fusion machinery have counterparts in other types of vesicle trafficking (Bennett and Scheller, 1993; Söllner and Rothman, 1994). For example, VAMP, syntaxin, and SNAP-25 all share homology with proteins that are essential for vesicle trafficking in yeast. These three proteins, or their homologes, are also involved in the docking and fusion events of membranes other than small synaptic vesicles in mammalian cells. If NO were truly stimulating neurotransmitter release by affecting the binding affinities of these core complex proteins, then it would not be surprising if other types of vesicle trafficking were similarly affected. Possibly the contents of not only small clear synaptic vesicles, but also dense core vesicles could be released in response to a NO signal. Since neuronal NO is released primarily in an activity-dependent fashion, this implies that release of a variety of other vesicle constituents, including growth factors, could be synchronized with activity. However, the extent to which these possible functions of NO are relevant in vivo remains to be seen.

Experimental Procedures

Materials and Chemicals

Clostridial neurotoxins were a gift from G. Schiavo and C. Montecucco. Rabbit anti-n-sec1, anti-SNAP 25, and anti-VAMP were provided by Jonathan Pevsner and Shu-Chan Hsu. Mouse anti-syntaxin (HPC-1) was purchased from Sigma. Chelex 100 resin was obtained from Bio-Rad, Percoll from Pharmacia, nitrocellulose from Schleicher and Schuell, and FM1-43 from Molecular Probes. Sucrose was obtained from J. T. Baker, S-nitroso-N-acetylpenicillamine (SNAP) from Research Biochemicals Incorporated, and ^{125}I-labeled secondary antibodies from Amersham, all other chemicals and reagents were from Sigma.

Isolation of Synaptosomes

Synaptosomes were isolated from the hippocampus by differential and discontinuous Percoll gradient centrifugations (Nagy and Delgado-Escueta, 1984), as described by Meffert et al. (1994).

Treatment with Clostridial Neurotoxins

In all BoNT experiments, synaptosomes in BSS plus 1 mM $CaCl_2$ were preincubated for 90 min at 37°C alone or in the presence of active or inactivated toxins. Synaptosomes were then cooled to 30°C before loading of FM1-43 (see below) was initiated with either KCl depolarization (40 mM) or a NO donor (500 μM). Synaptosomes were then stored on ice and fluorescence measurements made as described below. BoNT F holotoxin was used at 150 nM, BoNT C holotoxin at 80 nM, and BoNT A holotoxin at 150 nM. These concentrations were chosen based on previous use of botulinum neurotoxins in synaptosomes (McMahon et al., 1992; Blasi et al., 1993b; Yamasaki et al., 1994). BoNT inactivation was accomplished by boiling for 10 min (Blasi et al., 1993b).

Loading of Synaptosomes with FM1-43

Synaptosomes were loaded with dye according to Meffert et al. (1994). In brief, aliquots of control and BoNT-treated synaptosomes (~0.3 mg of protein/ml) were resuspended in BSS plus 1 mM calcium chloride and loaded with 5 μM FM1-43 for 10 min at 30°C followed by the addition of 40 mM KCl for 1 min. Following loading, synaptosomes were pelleted by brief centrifugation followed by washing, repelleting, and resuspending in BSS plus 1 mM $CaCl_2$. Fluorescence measurements were carried out as described by Meffert et al. (1994).

In Vitro-Binding Assays

The cytoplasmic domain of syntaxin 1a, full length n-sec1, soluble SNAP 25, or full-length VAMP2 were purified from recombinant fusion proteins as previously described (Pevsner et al., 1994b). Binding incubations consisted of syntaxin 1a/GST (0.3–0.7 μM) bound to glutathione beads (4 μl), and the indicated amount of recombinant protein. Samples were treated either with or without (control) a NO donor in buffer A containing 2 mM EDTA in place of 2.5 mM calcium chloride and incubated for 30 min at 21°C. NO donors were prepared in deoxygenated solutions and delivered using gas tight chromatography syringes. Following binding, beads were washed three times with 500 μl of buffer A containing 5% [v/v] glycerol followed by brief centrifugation. All washes were carried out at 4°C and were perfomed as quickly as possible to minimize disassociation. In NO treated samples, the first two washes also contained the same concentration of NO that was present during the binding period. In experiments using NEM, the NEM (3 mM) was added during the binding incubation in the same manner as the NO donor experiments. In β-ME reversal studies, the β-ME (500 μM) was added during the last 5 min of the binding incubation and in the first two washes; NO was not included in any of these washes (including all controls), and experiments were kept small to minimize wash times. Proteins on the beads were solubilized in 10 μl sample buffer and

subjected to electrophoresis and Western blotting (Pevsner et al., 1994b) with visualization and quantitation by autoradiography of ^{125}I-labeled goat anti-rabbit or anti-mouse secondary antisera and phosphoimaging (Molecular Dynamics or Fuji).

For saturation experiments, the EC_{50} was defined as half-maximal binding of each soluble protein based on pixel intensity obtained by phosphorimaging. EC_{50} was reported because K_D measurements are not valid at the high concentrations of proteins used (Bennett and Yamamura, 1985). The EC_{50} was estimated from plots of pixels versus concentration of protein added to beads (Pevsner et al., 1994b).

SDS-Resistant Complex

Formation of the high molecular weight SDS-resistant complex was carried out as described in Hayashi et al., 1994. Briefly synaptosomes or finely diced hippocampi were prepared in BSS plus 1 mM EGTA on ice, exposed to a NO donor (or no donor as control) for 5 min at 21°C, and then homogenized in a modified sample buffer containing a reduced amount of SDS: 60 mM Tris-HCl (pH 6.75), 5% v/v β-ME, 2% w/v SDS, 10% w/v glycerol, 0.007% w/v bromophenol blue. Aliquots of the homongenate were then diluted further into the modified sample buffer. Protein concentrations were quantified using the Bradford method and ~8 μg protein were loaded per gel lane. Duplicate samples were then either boiled (results in complex disassociation) or incubated at 37°C for 5 min. Samples were electrophoresed on 12% acrylamide gels at 4°C; Western blotting was carried out as described above, and the SDS-resistant complex was detected with anti-VAMP antibodies.

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